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Elimination of Micronuclear Specific DNA Sequences Early in Anlagen Development

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After conjugation in *Tetrahymena thermophila*, the old macronuclei degenerate, and new macronuclei (anlagen) develop. During anlagen development a number of DNA sequences found in the micronuclear genome (micronuclear limited sequences) are eliminated from the anlagen. A cloned copy of a repetitive micronuclear limited sequence has been used to determine the developmental stage at which micronuclear limited sequences are eliminated. DNAs from anlagen of various developmental stages were examined by Southern analysis. It was found that micronuclear limited sequences are present in 4C anlagen and essentially absent in 8C and 16C anlagen. The precipitous loss of these sequences in the 8C anlagen rules out under-replication as the mechanism for the loss and suggests that these sequences are specifically degraded early during anlagen development.

The ciliates are characterized by having both a germ line nucleus (micronucleus) and a somatic nucleus (macronucleus) in a common cytoplasm (12, 18). The macronucleus is responsible for virtually all gene expression during the vegetative growth (13, 25). In *Tetrahymena thermophila* the complexity of the macronuclear DNA is only about 80% that found in the micronucleus (15, 30). When cells enter the sexual cycle the old macronucleus degenerates, and a new macronucleus develops from division products of the new zygotic micronucleus (8, 22). During the development of the new macronuclei (anlagen), specific DNA sequences are lost (micronuclear limited sequences). A number of these micronuclear limited sequences have been characterized (3, 28). These eliminated sequences include both repetitive and unique sequences (3).

To investigate the process by which these sequences are eliminated, it is important to establish the stage during anlagen development at which elimination occurs. In the hypotrichous ciliates the elimination of micronuclear sequences during macronuclear development is extensive (17, 21). In these cells there is apparently a replication of the entire micronuclear genome followed by a degradation of the eliminated sequences and finally a further replication of the macronuclear sequences (1). In *T. thermophila* the developing anlagen progress through a series of endoreplications with the DNA content increasing in roughly a geometric fashion. The final result of this endoreplication is a macronuclear DNA content, immediately after conjugation, that is about 30% greater than that found in logarithmic growing cells. This slightly increased macronuclear DNA content usually persists for about 50 generations after conjugation (6, 7). Such a pattern is suggestive of the hypotrich elimination. It might be expected that all of the micronuclear sequences in *T. thermophila* are initially replicated during anlagen development and that the eliminated sequences are removed gradually during the first 50 generations after conjugation.

Other lines of evidence suggest that genome reorganization and the elimination of sequences may occur early in anlagen development. The ribosomal DNA in the micronucleus exists as a single copy integrated into the genome (29). In the macronucleus the ribosomal DNA exists as several thousand palindromic extrachromosomal ribosomal DNA molecules (9–11). The production of extrachromosomal ribosomal DNA molecules begins about 12 h after the initiation of conjugation (19, 20). This process involves both reorganization and elimination of DNA sequences (16, 27).

Yokoyama and Yao have presented autoradiographic evidence suggesting that DNA sequences are eliminated from anlagen during development (31). Using in situ hybridization, they report a modest replication of the micronuclear limited sequences before their elimination. In the Yokoyama and Yao study the elimination of sequences apparently occurs abruptly during early anlagen development.

The manner in which sequences are eliminated is unclear. During endoreplication of the developing anlagen, under-replication of specific sequences could lead to their elimination by dilution. Alternatively, sequences could be actively eliminated by specific degradation at a certain stage of anlagen development, as suggested by the autoradiographic data (31). The kinetics by which sequences are eliminated should distinguish between these possibilities.

In our study we have used a cloned repetitive micronuclear limited DNA sequence as a probe to determine the stage of anlagen development during which these sequences are eliminated. The probe sequences are clearly eliminated early during anlagen development, and the kinetics of elimination strongly suggests degradation as a mechanism, rather than under-replication and dilution.

MATERIALS AND METHODS

The strains of *T. thermophila* used were CU355 (mating type IV) and CU401 (mating type VII), provided by P. Bruns. CU355 is a heterocaryon, homozygous for cycloheximide resistance in the micronucleus, and phenotypically cycloheximide sensitive (4). The cells were grown to a relatively high cell density (5×10^5 to 8×10^5 cells per ml) in 1% proteose peptone (Difco Laboratories) and 0.1% liver extract L (Nutritional Biochemical Corp.) at 30°C with shaking, collected by gentle centrifugation (200 × *g* for 2 min) and suspended in 10 mM Tris (pH 7.5) at a cell density of 2.5×10^5 cells per ml. The cells of the two mating types were starved separately in 10 mM Tris for 6 to 12 h at 30°C with shaking. Conjugation was induced by mixing cells of the two different mating types and incubating without shaking (5). The percentage of true conjugants present in each mating

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was determined by cloning cells from the mating, allowing colonies to form, and challenging these colonies with cycloheximide (20 μg/ml). The parent strains are sensitive to cycloheximide; thus only cells completing conjugation were resistant to cycloheximide. In these experiments, 75 to 85% of the cells were true exconjugants.

Cells were harvested at different stages of conjugation by centrifugation. Cells containing predominately 4C anlagen were harvested at 12 h after initiation of conjugation, and cells with predominately 8C anlagen were harvested at 18 h. Cells with 16C anlagen were harvested at 22 h after initiation of conjugation from cells that were refed at 15 h after conjugation. Cells must be refed for anlagen development to progress substantially beyond the 8C stage (Brunk and Bohman, manuscript in preparation).

Approximately 1.5×10^8 cells were harvested and suspended in 25 ml of homogenization buffer (10 mM Tris [pH 7.5], 4% gum arabic, 3% sucrose, 5 mM spermidine, 1 mM spermine [14]). Cells were blended in a Sorvall homogenizer at full speed for 30 s immediately after the addition of 1% octanol. The homogenate was diluted to 500 ml with homogenization buffer, mixed well, and centrifuged at $200 \times g$ for 25 min to remove large cellular debris. The supernatant was centrifuged at $5,000 \times g$ for 20 min, and the pellet containing the nuclei was suspended in 25 ml of homogenization buffer and loaded on top of a 250-ml 10 to 45% sucrose gradient containing the ingredients of the homogenization buffer. The gradient was centrifuged at $200 \times g$ for 50 min, and fractionated into 25-ml samples, which were then centrifuged at $10,000 \times g$ for 10 min to pellet the nuclei. The nuclei were suspended in 2 ml of homogenization buffer and examined after staining with 4′,6-diamidino-2-phenylindole 2-hydrochloride (0.1 μg/mg) with fluorescent microscopy (λ = 300 nm). Fractions enriched in anlagen were pooled and further purified on an additional gradient and analyzed.

The nuclear content of the final anlagen fractions was determined by flow cytometer analysis of samples stained with propidium iodide (25 μg/ml) with a Coulter EPIC V flow cytometer. Pooled nuclei were centrifuged and washed in homogenization buffer without gum arabic, and DNA was prepared from the nuclei (2).

DNA from the various fractions was digested with *Hin*dIII restriction endonuclease, separated by gel electrophoresis, transferred to BA85 nitrocellulose (Schleicher & Schuell Co.), and hybridized with radioactive DNA in a Southern analysis (24). The DNA used as a probe in these experiments was purified by agarose gel electrophoresis, removed from the gel by electroelution onto NA45 DEAE membrane (Schleicher & Schuell) as described by the supplier, and labeled to a specific activity of 5×10^7 to 10×10^7 cpm/μg with [α-^{32}P]dATP and [α-^{32}P]dCTP (Amersham) by nick translation (23). Enzymes were obtained from Bethesda Research Laboratories, Inc., and New England Biolabs, Inc., and used as prescribed by the suppliers.

Photographs of ethidium bromide (0.5 μg/ml)-stained gels and Southern analyses were scanned with a microdensitometer (Joyce-Loebl) to quantitate the amount of DNA and the amount of hybridization. The amount of hybridization was normalized by the amount of DNA on the gel. The contribution to the final DNA content made by each of the various types of nuclei was calculated from the relative abundance of the various nuclear types (flow cytometry data) and the relative amount of DNA in each nuclear type. The relative amounts of DNA in the nuclei were as follows: micronuclei, 1; 4C anlagen, 1; 8C anlagen, 2; 16C anlagen, 4; and macronuclei, 10. A comparison of the probe hybridization to anlagen-enriched fractions and micronucleus-enriched fractions allows one to calculate the amount of that specific sequence still present in the anlagen. The amount of our micronuclear limited sequence present in the anlagen is expressed as a percentage of the amount present in the micronuclei.

RESULTS

We have established the time at which DNA sequences are eliminated from the developing anlagen by using a micronuclear limited sequence as a probe and determining the stage at which these sequences can no longer be detected by hybridization. We chose as a convenient probe a 450-base-pair sequence between an *Xba*I and an *Hin*dIII restriction site (X-H) cloned from micronuclear DNA (3). Figure 1 shows hybridization of the X-H probe with both macronuclear and micronuclear DNA digested with either *Eco*RI or *Hin*dIII endonuclease. Even with an extended exposure there is no detectable hybridization between this probe and macronuclear DNA. This sequence is repetitive in the micronucleus, but has undetectable hybridization with macronuclear DNA.

After conjugation we found that the developing anlagen could progress to about the 8C stage without refeeding; further anlagen development required refeeding. When nuclei were prepared from cells at 12 h after conjugation and analyzed with the flow cytometer, the predominate species of anlagen present was 4C. These anlagen had the same

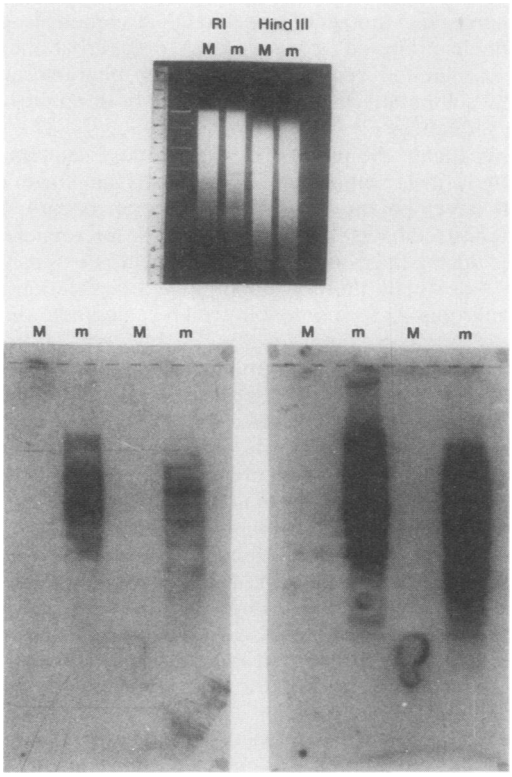

FIG. 1. Southern analysis of macronuclear and micronuclear DNA probed with X-H sequence. The ethidium bromide-stained gel (top) shows macronuclear DNA (M) and micronuclear DNA (m) restricted with *Eco*RI (RI) or *Hin*dIII. The autoradiograms (bottom) were probed with the X-H sequence and exposed for 45 h (left) or overexposed for 240 h (right).

amount of DNA as the micronuclei, but the volume of the anlagen was much greater than that of the micronuclei. The flow cytometry diagram shown in Fig. 2a displays fluorescence on one axis (left to right) and forward-angle light scatter on another axis (front to back) with the numbers of nuclei on the third axis (vertically). Fluorescence is a direct measure of the DNA content (displayed on a logarithmic scale), whereas the forward-angle light scatter is related to the nuclear volume. The relatively sharp peak of nuclei at the lower light scatter values represents the micronuclei, whereas the anlagen can be seen as a broader peak at higher light scatter values. In *T. thermophila* virtually all of the micronuclei are in the G2 portion of the cell cycle and thus have a 4C DNA content (26). These anlagen are clearly 4C anlagen, since they have the same value of fluorescence or DNA content as micronuclei. In this display of 12-h anlagen the vast majority of anlagen are 4C anlagen.

A similar flow cytometer diagram for nuclei isolated from cells at 18 h after conjugation is shown in Fig. 2b. The micronuclear peak appears in a similar position, but now the anlagen peak is not only larger in volume (higher light scatter values), but also has fluorescence value twice the micronuclear value. These 18-h anlagen are virtually all 8C.

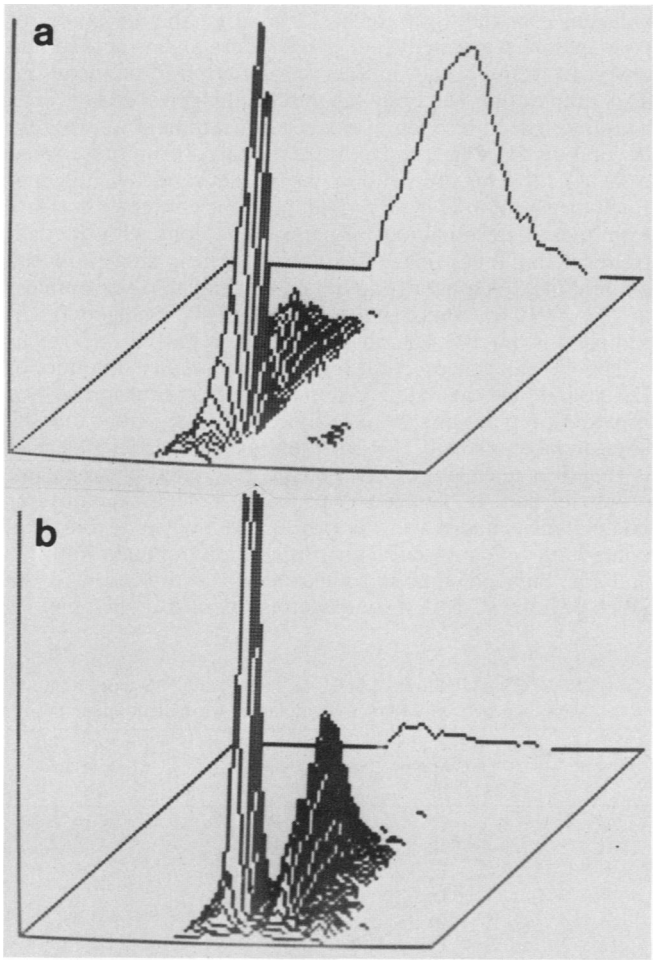

FIG. 2. Flow cytometric analysis of the nuclei from cells during anlagen development. The DNA content, measured by propidium iodide fluorescence, is displayed on a logarithmic scale left to right. The relative nuclear size, measured by forward-angle light scatter, is displayed front to back. The number of nuclei are displayed vertically. (a) Nuclei from cells 12 h after the initiation of conjugation. The high peak in the foreground is micronuclei, with 4C anlagen directly behind. (b) Nuclei from cells 18 h after the initiation of conjugation. Again the high peak is micronuclei, with 8C anlagen to the right and behind.

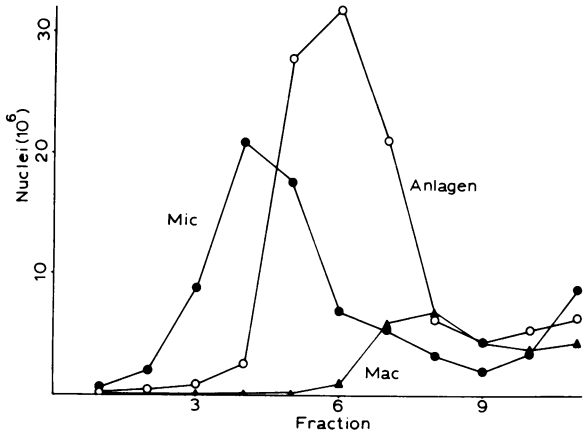

FIG. 3. Distribution of nuclei, isolated from cells 18 h after the initiation of conjugation, on a sucrose gradient. The relative amounts of the various nuclei types were determined by fluorescent microscopic analysis. The anlagen are predominately 8C. Mic, Micronuclei; Mac, macronuclei.

The old macronuclei degenerate after conjugation. In the 12-h sample many of the macronuclei have been lost, and those remaining are probably represented by the trace at limit of the forward light scatter range (Fig. 2a). By 18 h most of the cells have lost their old macronuclei, and those that remain are severely picnotic (fluorescence microscope data, not shown).

The initial determination of sequence elimination was performed on nuclei isolated from cells 18 h after the initiation of conjugation. These cells contain micronuclei and 8C anlagen in equal numbers plus a small number of degenerating macronuclei. After homogenization and a brief differential sedimentation, the nuclei were placed on a 10 to 45% sucrose gradient. Each gradient was fractionated into about 10 fractions. The nuclear composition of each fraction was estimated by fluorescence microscopy, and fractions enriched in micronuclei or anlagen were pooled and further fractionated on an additional gradient. Figure 3 shows the nuclear composition of such a gradient used to fractionate nuclei from cells 18 h after conjugation (8C anlagen). The nuclear percentages of various fractions enriched for micronuclei or anlagen were established with the flow cytometer (greater than 10^4 nuclei per sample; error less than 1%). The sucrose gradient fractionation provides a reasonable degree of enrichment for micronuclei and anlagen.

DNA was prepared from each fraction and purified by CsCl gradient centrifugation. This DNA was digested with *Hin*dIII and subjected to gel electrophoresis and Southern analysis with the cloned X-H sequence as a probe. The stained gel and the corresponding Southern analysis of fractions are shown in Fig. 4. A photograph of the stained gel and an autoradiogram of the Southern analysis were scanned with a microdensitometer, and the amount of hybridization was quantitated. Independent scans as well as control hybridizations indicate that the error in estimating the relative hybridization is less than 20%. Table 1 shows the amount of radioactive probe that hybridized to the sample DNA normalized by the amount of DNA on the gel. The relative percentages of micronuclei, anlagen, and macronuclei in each fraction as well as the respective percentages of DNA

are also shown. The micronuclear sample is a pool of fractions enriched in micronuclei, whereas fractions 5 through 7 are individual fractions that have progressively higher percentages of 8C anlagen.

The macronuclear DNA does not contain sequences homologous to the probe; thus only the micronuclear and anlagen DNA have homologous sequences. Each anlagen-enriched fraction can be compared with the micronuclear sample to yield a simultaneous solution for the relative amount of hybridization to the anlagen and micronuclear DNA. These values, expressed as the percentage of X-H probe hybridization to 8C anlagen DNA relative to the hybridization to micronuclear DNA, are shown in the last column of Table 1. The amount of hybridization to anlagen DNA ranges from 8 to 14% of the amount of hybridization found in micronuclear DNA. The two samples containing the highest anlagen contents both indicate 8% of the amount of hybridization that is found in micronuclear DNA. Any pair of samples can be used for an estimate, but the best accuracy is achieved in comparisons of samples containing predominately micronuclear DNA with samples containing predominately anlagen DNA. The 8C anlagen DNA has on average 10% of the amount of hybridization that is found in micronuclear DNA. Given the inherent variability in this type of measurement, it is hard to attach much significance to this small amount. Clearly the majority of the X-H sequences are eliminated from the 8C anlagen.

FIG. 4. Southern analysis of fraction from a sucrose gradient separation of micronuclei and anlagen. The ethidium bromide-stained gel (top) shows a fraction containing predominately micronuclei (M) and several fractions containing predominately anlagen (lanes 5, 6, and 7). The autoradiogram (bottom) was probed with the X-H sequence and exposed for 48 h.

TABLE 1. Hybridization of X-H sequence to 8C anlagen

Fraction	Hybridization/DNA[a]	% of nuclei (DNA)			% Hybridization 8C[b]
		Micronuclei	8C	Macronuclei	
Micronuclei	1.03	86 (71)	13 (21)	1 (8)	
5	0.66	59 (40)	40 (54)	1 (6)	14
6	0.34	36 (19)	61 (65)	3 (16)	8
7	0.23	28 (12)	64 (54)	8 (34)	8

[a] Amount of hybridization detected by Southern analysis normalized to the amount of DNA on the gel.

[b] The calculated value for hybridization to anlagen DNA expressed as a percentage of hybridization to micronuclear DNA.

The procedure described above was also used to determine the amount of X-H sequence present in other stages of anlagen development. Two separate preparations of 4C anlagen, isolated from cells 12 h after the initiation of conjugation, were analyzed. Nuclei were also prepared and analyzed from cells 16 h after the initiation of conjugation. This preparation has both 4C and 8C anlagen. Finally, cells were refed at 15 h to allow anlagen development to progress beyond the 8C stage. Nuclei were prepared from these refed cells at 22 h. This preparation has both 8C and 16C anlagen. Each preparation is from an independent conjugation. Data from these determinations are presented along with the data from the initial 8C anlagen in Table 2. These data show the amount of X-H sequence found in 4C anlagen (five samples) is 103 ± 10%, whereas that found in 8C anlagen (eight samples) is 6.6 ± 3.4%, and 16C anlagen have 3.3 ± 1.6%.

The 4C anlagen preparations contain smaller amounts of DNA, and thus the data are somewhat less accurate, so two separate preparations were analyzed. It is apparent that 4C anlagen have virtually the same amount of X-H sequence as is found in micronuclei. The slight difference between micronuclei and 4C anlagen is probably not significant. For completeness nuclei were prepared and analyzed from cells containing 16C anlagen. This preparation contains both 8C and 16C anlagen; thus information on hybridization to the DNA of both 8C and 16C anlagen is obtained. The analysis

TABLE 2. Hybridization of X-H sequence to DNA from anlagen expressed as a percentage of hybridization to micronuclear DNA

Anlagen	Time after conjugation (h)	Fraction	% Hybridization
4C	12	A	105
4C	12	B	107
4C	12	A	119
4C	16	A	97
4C	16	B	89
8C	16	A	3
8C	16	B	7
8C	18	5	14
8C	18	6	8
8C	18	7	8
8C	22	A	6
8C	22	B	3
8C	22	C	4
16C	22	A	2
16C	22	B	5
16C	22	C	3

indicates that the X-H sequence is absent from the 16C anlagen as well as the 8C anlagen.

An analysis of nuclei prepared from cells 16 h after the initiation of conjugation indicates that the relative amounts of X-H sequence in 4C and 8C anlagen are similar to the previous results. The X-H sequence is present in 4C anlagen in amounts equivalent to that found in micronuclei and absent in 8C anlagen. Even these relatively new 8C anlagen have the X-H sequence eliminated.

The amount of X-H sequence present in the different stages clearly indicates that this sequence is present in 4C anlagen in an amount similar to that in micronuclei and virtually absent in 8C anlagen. This precipitous loss of sequences indicates that the elimination occurs by an active degradation of these sequences rather than by an under-replication. An under-replication would lead to a halving of the amount of the sequence at each replication. The loss observed is apparently much more rapid than a halving between 4C and 8C anlagen.

DISCUSSION

The results reported here help to establish two important facts regarding the elimination of sequences during anlagen development. First, the timing of sequence elimination is early in anlagen development. Second, these eliminated sequences must be removed by active degradation rather than by passive under-replication and dilution.

The elimination of the X-H sequence used as a probe in these studies occurs between the 4C and 8C stages. The results reported here indicate that the amount of X-H sequence present in 4C anlagen is similar to that found in micronuclei, whereas the X-H sequence is virtually absent from the 8C (and 16C) anlagen. Even early 8C anlagen apparently have lost the X-H sequences.

The eliminated sequences are most likely removed by an active degradative process. Under replication would reduce the amount of X-H sequence found in 8C anlagen to one half the amount found in 4C anlagen (or in micronuclei). The results reported here indicate that few or no X-H sequences are found in 8C anlagen. This precludes under-replication and dilution as a mechanism for sequence elimination during anlagen development.

The rapid elimination of sequences between two stages implies that specific nucleases are most probably involved. It is also most probable that sequences at the boundaries of the eliminated sequences are specifically recognized. It is also of interest that the multiple copies of the X-H sequence found the micronuclear genome are virtually all eliminated between the 4C and 8C stage. This coordinated removal of micronuclear limited sequences implies that a precise developmental process is responsible for the elimination.

Our results indicate that there is not a significant amount of replication of the X-H sequences before their elimination. This is somewhat at variance with the results reported by Yokoyama and Yao (31). They suggest that the sequences they examined were replicated about threefold before elimination. Given that the timing of the elimination in both studies is before the 8C stage, it is unlikely that much replication of the eliminated sequences occurs. However, different sequences were examined in these studies, and it is possible that different amounts of replication occur for different sequences before elimination. Alternatively, in situ hybridization to enlarged anlagen might be more efficient than to micronuclei, giving the impression that replication of the eliminated sequences had occurred before their removal.

The timing we have established for the elimination of these sequences correlates well with the autoradiographic study of Yokoyama and Yao on sequence elimination (31). The formation of extrachromosomal ribosomal DNA in developing anlagen also apparently occurs at this time (19, 20). Thus, the formation of 8C anlagen at 14 to 18 h after the initiation of conjugation is the time at which sequence elimination and reorganization in developing anlagen occur.

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